Federal Democratic Republic of Ethiopia

EDICT OF GOVERNMENT

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ET ISO 8870 (2012) (English): Milk and milk-based products - Detection of thermonuclease produced by coagulase-positive staphylococci

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ETHIOPIAN
STANDARD

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First edition

Milk and milk-based products — Detection of thermonuclease produced by coagulase-positive staphylococci

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Foreword

This Ethiopian Standard has been prepared under the direction of the Technical Committee for Milk and Milk Products (TC 25) and published by the Ethiopian Standards Agency (ESA). The standard is identical with ISO 8870 :2006 Milk and milk-based products — Detection of thermonuclease producedby coagulase-positive staphylococci published by the International Organization for Standardization, ISO 2006.

For the purpose of this Ethiopian Standard, the adopted text shall be modified as follows.

- The phrase “International Standard” shall be read as “Ethiopian Standard”; and
- A full stop (.) shall substitute comma (,) as decimal marker.

Milk and milk-based products — Detection of thermonuclease produced by coagulase-positive staphylococci

1 Scope

This International Standard specifies a method for the detection of heat-stable DNase (thermonuclease) produced by coagulase-positive staphylococci in milk and milk-based products.

The enzyme can be used as an indicator that staphylococcal growth has reached hazardous levels, and can reveal the potential presence of staphylococcal enterotoxins.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 8261|IDF 122, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
thermonuclease
enzyme produced by coagulase-positive staphylococci, which hydrolyses deoxyribonucleic acid (DNA) under the conditions specified in this International Standard

4 Principle

Milk, reconstituted milk powder or homogenized milk-based products are, after addition of skim milk powder, adjusted to pH 3,8 and centrifuged. The supernatant is precipitated with trichloroacetic acid. After centrifuging, the precipitate is adjusted to pH 8,5 and Tris buffer is added. The solution is then heated in a boiling water bath for 15 min and tested for thermonuclease activity in toluidine blue O-DNA agar. The colour of the agar turns from blue to pink if thermonuclease cleaves the DNA molecule. This colour reaction is due to the metachromatic properties of toluidine blue O.

5 Culture media and reagents

5.1 Basic materials

For current laboratory practice, see ISO 8261|IDF 122.

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

If the prepared media and reagents are not used immediately, store them, unless otherwise stated, in the dark at between 0 °C and +5 °C for no longer than 1 month, under conditions which do not produce any change in their composition.

5.2 Toluidine blue O-DNA agar

5.2.1 Tris buffer

5.2.1.1 Composition

Tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$)	6,06 g
Calcium chloride ($CaCl_2$)	0,11 g
Water	1 000 ml

5.2.1.2 Preparation

Dissolve the tris(hydroxymethyl)aminomethane and the calcium chloride in 980 ml of water. Adjust the pH to 9,0 and add water to 1 000 ml.

5.2.2 Base medium

5.2.2.1 Composition

Deoxyribonucleic acid (DNA)[a]	0,3 g
Sodium chloride (NaCl)	10,0 g
Agar	10,0 g
Tris buffer (5.2.1)	1 000 ml

[a] The DNA used shall have been proven to be suitable for the detection of thermonuclease.

5.2.2.2 Preparation

Boil the components in a water bath (6.2) until the DNA and the agar are completely dissolved (about 1,5 h).

5.2.3 Toluidine blue O solution

5.2.3.1 Composition

Toluidine blue O ($C_{15}H_{16}N_3SCl$)[a]	0,31 g
Water	10 ml

[a] Toluidine blue O (Colour Index: C.I. Basic Blue 17; C.I. No. 52040).

5.2.3.2 Preparation

Dissolve the toluidine blue O in the water with minimal heating and filter it through gauze (6.12).

5.2.4 Complete medium

5.2.4.1 Composition

Base medium (5.2.2)	1 000 ml
Toluidine blue O solution (5.2.3)	3 ml

5.2.4.2 Preparation

Cool the base medium to approximately 50 °C. Add the filtered toluidine blue O solution and mix by swirling.

The complete medium may be stored for several months in small portions in closed flasks or bottles in the dark at between 0 °C and +5 °C.

5.2.5 Preparation of agar plates

Transfer to Petri dishes (6.8), 13 ml of the complete medium (5.2.4) or the stored medium preheated to approximately 50 °C. Allow it to solidify.

Prepared plates may be stored for about 2 months (bottom uppermost and protected from drying) in the dark at between 0 °C and +5 °C.

5.3 Hydrochloric acid, $c(\mathrm{HCl}) = 2$ mol/l.

5.4 Sodium hydroxide, $c(\mathrm{NaOH}) = 2$ mol/l.

5.5 Trichloroacetic acid, $c(\mathrm{CCl_3COOH}) = 3$ mol/l.

Dissolve 49,02 g of trichloroacetic acid in 100 ml of water.

5.6 Skim milk powder

Skim milk powder shall be free from thermonuclease. Test every new lot using the method described in this International Standard.

5.7 Brain heart infusion broth

5.7.1 Composition

Enzymatic digest of animal tissues	10,0 g
Dehydrated calf brain infusion	12,5 g
Dehydrated beef heart infusion	5,0 g
Glucose	2,0 g
Sodium chloride	5,0 g
Disodium hydrogen phosphate, anhydrous ($\mathrm{Na_2HPO_4}$)	2,5 g
Water	1 000 ml

5.7.2 Preparation

Dissolve the components or the dehydrated complete medium in the water by heating, if necessary. Adjust the pH so that after sterilization it is 7,4 ± 0,2 at 25 °C. Transfer the culture medium in quantities of approximately 5 ml in tubes of appropriate capacity (6.11).

Sterilize the medium at 121 °C for 15 min.

5.8 Test microorganism (positive control)

Any thermonuclease positive strain of *Staphylococcus aureus* may be used as test microorganism.

6 Apparatus

Use the apparatus required for the preparation of test samples as specified in ISO 8261|IDF 122, the usual microbiological laboratory apparatus and, in particular, the following.

Disposable apparatus is an acceptable alternative to reusable glassware if it has suitable specifications. Reusable glassware should be capable of undergoing repeated sterilization and should be chemically inert.

6.1 Incubator, capable of being maintained at 37 °C ± 1 °C.

6.2 Water baths, capable of operating at 50 °C and of boiling (for heating and cooling of the medium and the solutions to suitable temperatures).

6.3 Centrifuge, capable of delivering a centrifugal force of more than 27 000 *g*.

6.4 Centrifuge, capable of delivering a centrifugal force of more than 2 800 *g*.

6.5 Hollow cylinder, made from metal, of diameter about 2 mm, for cutting wells into the toluidine blue O-DNA agar.

6.6 Micropipettes, of nominal capacity 0,01 ml.

6.7 Loops, of platinum-iridium or nickel-chromium, of diameter approximately 3 mm.

6.8 Petri dishes, made of glass or plastic, of diameter between 90 mm and 100 mm.

6.9 Measuring cylinders, of nominal capacities 10 ml, 100 ml and 1 000 ml.

6.10 Bottles, with stoppers, for storing toluidine blue O-DNA agar.

6.11 Test tubes, of diameter 15 mm and length 100 mm.

6.12 Gauze (for example absorbent cotton gauze Ph. Eur. type 20)1).

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 707|IDF 50.

Store test samples in such a way that deterioration and change in composition are prevented.

8 Procedure

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1) Absorbent cotton gauze Ph. Eur. type 2 is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO or IDF of this product.

8.1 Preparation of the test sample

8.1.1 Milk

Adjust 100 ml of test sample to pH 3,8 with hydrochloric acid (5.3).

8.1.2 Milk-based products

Suspend 20 g of test sample and 5 g of skim milk powder (5.6) in 50 ml of water. Adjust the pH to 3,8 with hydrochloric acid (5.3).

The addition of skim milk powder is not necessary for all milk-based products. It may be omitted, for instance, when skim milk powder, whole milk powder or caseins are examined. The addition is required in the case of whey powder, yoghurt and fruit yoghurt and fruit ice cream. For any other milk-based products, the addition of skim milk powder is recommended.

For the examination of cheese, the following extraction procedure may be used as an alternative. Suspend 20 g of cheese in 100 ml of water (at 45 °C) in a blender. After blending, adjust the pH to 4,5 using hydrochloric acid (6 mol/l). Transfer the suspension to a 750 ml flat-bottom flask. Add water up to a final mass of 12,5 times the cheese mass. Agitate the suspension overnight in a water bath set at 25 °C for 16 h. Adjust the pH to 4,5 again. Centrifuge and filtrate the suspension. Continue the procedure as described in 8.1.3 with 100 ml of filtrate.

If skim milk powder is not added, suspend 20 g of test sample in 40 ml water. Adjust the pH to 3,8 with hydrochloric acid (5.3).

8.1.3 General procedure

8.1.3.1 Centrifuge (6.3) the test sample at between 27 000 *g* and 34 000 *g* at 5 °C for 20 min.

8.1.3.2 Decant the supernatant. Add 0,05 ml of cold trichloroacetic acid (5.5) for each millilitre of sample and mix.

8.1.3.3 Centrifuge at between 27 000 *g* and 34 000 *g* at 5 °C for 20 min.

8.1.3.4 Dissolve the sediment in 1 ml of Tris buffer (5.2.1). Adjust the pH to 8,5 with sodium hydroxide (5.4). Dilute the solution to 2 ml with Tris buffer and mix.

8.1.3.5 Heat the solution in a boiling water bath (6.2) for 15 min.

8.2 Preparation of the test microorganism (positive control)

8.2.1 Inoculate the brain heart infusion broth (5.7) with the test microorganism (5.8). Incubate at 37 °C for 24 h. Centrifuge (6.4) at between 2 800 *g* and 3 500 *g* for 15 min. Decant the supernatant.

8.2.2 Heat the supernatant in a boiling water bath (6.2) for 15 min.

The heated supernatant may be stored at 5 °C for about 4 weeks.

If necessary, any *Staphylococcus aureus* strain (for example isolated from milk or milk-based products) may be tested for thermonuclease production in the same way as described for the test microorganism.

8.3 Preparation of the toluidine blue O-DNA agar

Using the hollow cylinder (6.5), cut 2 wells in the agar (5.2.5). Dry the plates, with the lids off and the agar surface downwards, in an incubator (6.1) set at 37 °C for about 60 min.

If there are several samples to be examined, cut up to 10 wells in the agar.

8.4 Detection of thermonuclease

8.4.1 Fill one of the wells with 10 µl of positive control (8.2.2) and the other well(s) with 10 µl (each) of test sample(s) (8.1.3.5).

8.4.2 Incubate (6.1) the Petri dishes (lids uppermost) at 37 °C for 4 h. If the result is negative after 4 h, incubate the dishes for up to 24 h.

9 Evaluation and interpretation

A pink halo extending 1 mm or more beyond the well indicates the presence of thermonuclease (refer to the positive control). Halos showing colours other than pink are not ascribed to thermonuclease activity.

A positive thermonuclease test indicates that coagulase-positive staphylococci have grown to levels of 10^6 or more per gram.

In the case of enterotoxin formation, such counts may lead to toxin concentrations high enough to cause illness. Therefore, products that are positive in the thermonuclease test should be tested for the presence of enterotoxins.

10 Test report

The test report shall specify:

a) all information required for the complete identification of the sample;

b) the sampling method used, if known;

c) the method used, with reference to this International Standard;

d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incident which may have influenced the result(s);

e) the test result(s) obtained and, if the repeatability has been checked, the final quoted results obtained.

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Bibliography

[1] ISO 707 | IDF 50, *Milk and milk products — Guidance on sampling*

[2] *Colour Index*, The Society of Dyers and Colourists, Bradford (England) and The American Association of Textile Chemists and Colorists, North Carolina (USA), 1971

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Ethiopian Standards Agency

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Organization and Objectives

The Ethiopian Standards Agency (ESA) is the national standards body of Ethiopia established in 2010 based on regulation No. 193/2010.ESA is established due to the restructuring of Quality and Standards Authority of Ethiopia (QSAE) which was established in 1970.

ESA's objectives are:-

- Develop Ethiopian standards and establish a system that enable to check weather goods and services are in compliance with the required standards,
- Facilitate the country's technology transfer through the use of standards,
- Develop national standards for local products and services so as to make them competitive in the international market.

Ethiopian Standards

The Ethiopian Standards are developed by national technical committees which are composed of different stakeholders consisting of educational Institutions, research institutes, government organizations, certification, inspection, and testing organizations, regulatory bodies, consumer association etc. The requirements and/ or recommendations contained in Ethiopian Standards are consensus based that reflects the interest of the TC representatives and also of comments received from the public and other sources. Ethiopian Standards are approved by the National Standardization Council and are kept under continuous review after publication and updated regularly to take account of latest scientific and technological changes. Orders for all Ethiopian Standards, International Standard and ASTM standards, including electronic versions, should be addressed to the Documentation and Publication Team at the Head office and Branch (Liaisons) offices. A catalogue of Ethiopian Standards is also available freely and can be accessed in from our website.

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International Involvement

ESA, representing Ethiopia, is a member of the International Organization for Standardization (ISO), and Codex Alimentarius Commission (CODEX). It also maintains close working relations with the international Electro-technical Commission (IEC) and American Society for Testing and Materials (ASTM).It is a founding member of the African Regional Organization for standardization (ARSO).

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